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Akt Signaling and Coordinated Changes in the Distribution and Expression of Akt-Regulating Phosphatases

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ABSTRACT

Cancer is one of the major causes of death worldwide. The PI3K/Akt signaling pathway is up-regulated in a variety of human cancers. Akt is an important signaling molecule in cellular survival pathways. Activated Akt (pAkt) is able to induce protein synthesis pathways, and is therefore a key protein involved in growth and prevention of apoptosis. Several lipid or protein phosphatases exist that inhibit Akt signaling. Nuclear localization of pAkt is crucial for its activity and function.

Previously, it was demonstrated that cholesterol-lowering and anti-carcinogenic drugs, statins, rapidly depleted nuclear pAkt. We focused on the mechanism behind this rapid nuclear pAkt depletion. In paper I our results showed that statins or extracellular ATP induced a complex and coordinated response in insulin-stimulated A549 cells leading to depletion of nuclear pAkt. This involved lipid/protein phosphatases PTEN, PHLPP1 and -2, PP2A and calcineurin. Purinergic P2X7 receptor was identified to be a mediator of this effect.

In study II, the rapid nuclear pAkt depletion was further investigated and the possible role of a PI3K subunit, p110β, was elucidated. This subunit has been associated with aggressive prostate cancer, and studies on mouse embryonic fibroblast cells and cancer cells showed that p110β is essential for nuclear pAkt depletion.

EHBP1 and P-Rex1 have been involved in protein transport and membrane recruitment of proteins, and both of these proteins have been associated with aggressive or invasive prostate cancer. In paper III we found that P2X7 correlated with aggressive prostate cancer and that P2X7-mediated rapid nuclear pAkt depletion is dependent of both EHBP1 and P-Rex1. Moreover, pharmacological concentrations of statins decreased nuclear pAkt in non-transformed prostatic cells, suggesting that the anticancer effect of statins might be mediated by inhibition of the Akt pathway.

In Paper IV we characterized crosstalk between PHLPPs and PTEN, two proteins that down-regulate Akt activity. This crosstalk was seen in cancer cells and TGFβ-1-activated prostate stem cells, and had an impact on cellular invasiveness. The P2X4 receptor was identified to be a mediator of crosstalk induction. Downstream of P2X4 epigenetic and transcriptional factors were activated.

Overall, these studies show a novel mechanism leading to nuclear pAkt depletion. We also provide evidence for a role of P2X7-EHBP1-Akt axis in prostate cancer development and that inhibition of Akt may affect the invasive capacity of the cancer cells. A crosstalk between Akt phosphatases regulates Akt and affects invasiveness.

INSTITUTE OF ENVIRONMENTAL MEDICINE

Karolinska Institutet, Stockholm, Sweden

AKT SIGNALING AND COORDINATED CHANGES IN THE DISTRIBUTION AND EXPRESSION OF AKT-REGULATING PHOSPHATASES

Aram Ghalali

Karolinska Institutet

Stockholm 2014

قەڵەم

قەڵەمێک سەری خۆی هەڵگرت
هیچ دەفتەر و هیچ کاغەزێ
نەیانزانی
رووی لەکوێ کرد
چونکە ویستیان دای بدەن و
خۆی بەخۆی بکوژێننەوە!!
چونکە ویستیان وابنوسێ
هەر بەتنیا سوڵتانەکان
بیخوێننەوە

شێرکۆ بێکەس

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This thesis is dedicated to both my parents.

In loving memory of my inspiring ***father*** *(R.I.P), the source of my strength and my moral compass.*

Even though I lost you at very young age, your words of encouragement and push for tenacity will ring in my ears for as long as I live.

And

For the devotion and care of my wonderful ***mother****.*

All I have and will accomplish are only possible due to your endless love, support and sacrifices.

(A. S. N. F)

“Precision is the master of all things, not as a goal in itself, but simply as a tool of performance, permeated by discipline”

Aram Ghalali

Human beings are members of a whole,

In creation of one essence and a soul,

If one member is afflicted with pain,

Other members uneasy will remain,

If you´ve no sympathy for human pain,

The name of human you cannot retain.

(Saadi Shirazi 1210 – 1291)

<u>Thesis defense</u>

Pharmacology Lecture Hall

Address: Nanna Svartz väg 2

Karolinska Institutet, Solna

Friday 23rd of May 2014 at 09:00

Scan me for the location!

ABSTRACT

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LIST OF SCIENTIFIC PUBLICATIONS

I. Mistafa O[*], **Ghalali A**[*#], Kadekar S, Högberg J, Stenius U.
Purinergic receptor-mediated rapid depletion of nuclear phosphorylated Akt depends on pleckstrin homology domain leucine-rich repeat phosphatase, calcineurin, protein phosphatase 2A, and PTEN phosphatases.
J Biol Chem. 2010 Sep 3;285(36):27900-10.

II. Ye ZW, **Ghalali A**, Högberg J, Stenius U.
Silencing p110β prevents rapid depletion of nuclear pAkt.
Biochem Biophys Res Commun. 2011 Dec 2;415(4):613-8.

III. **Ghalali A**, Wiklund F, Zheng H, Stenius U, Högberg J.
Atorvastatin prevents ATP-driven invasiveness via P2X7 and EHBP1 signaling in PTEN-expressing prostate cancer cells.
Carcinogenesis. 2014 Feb 17;10.1093/carcin/bgu019

IV. **Ghalali A**[#], Ye ZW, Högberg J, Stenius U.
Phosphatase and Tensin Homolog Deleted on Chromosome 10 (PTEN) and PH Domain and Leucine-rich Repeat Phosphatase Cross-talk (PHLPP) in Cancer Cells and in Transforming Growth Factor β-Activated Stem Cells
J Biol Chem. 2014 Apr 25; 289(17):11601-15.

***** Both authors contributed equally to this work

Corresponding author

ADDITIONAL PUBLICATION, NOT INCLUDED IN THE THESIS

Karim H, **Ghalali A**, Lafolie P, Vitols S, Fotoohi AK.
Differential role of thiopurine methyltransferase in the cytotoxic effects of 6-mercaptopurine and 6-thioguanine on human leukemia cells.
Biochem Biophys Res Commun. 2013 Jul 26;437(2):280-6.

CONTENTS

LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
CD-PTEN	C-terminus deleted PTEN
CGEMS	Cancer Genetic Markers of Susceptibility
EHBP1	EH domain-binding protein 1
EMT	Epithelial-mesenchymal transition
FKBP51	FK506-binding protein 51
GLUT4	Glucose transporter type 4
HEK293	Human Embryonic Kidney 293 cells
HMG-CoA	3-hydroxy-3-methyl-glutaryl-coenzyme A reductase
ILK	Integrin-linked kinase
MEF	Mouse embryonic fibroblasts
MMP2	Matrix metalloprotease-2
MMP9	Matrix metalloprotease-9
miR	Micro-RNA or micro ribonucleic acid
mRNA	Messenger RNA or messenger ribonucleic acid
MST1	Mammalian sterile 20-like kinase-1
mTORC2	Mammalian target of rapamycin Complex 2
NEDD4-1	Neural precursor expressed developmentally downregulated 4-1
NFAT	Nuclear factor of activated T-cells
NFκβ	Nuclear factor-κβ
NHERF	Na+/H+ exchanger regulatory factor 1
NLS	nuclear localization signal
pAkt	Phosphorylated Akt
PcG	Polycomb group of proteins
PCNA	Proliferating cell nuclear antigen
PDK1	Phosphoinositide-dependent protein kinase 1
PH	Pleckstrin homology domain
PHLPP	Pleckstrin homology domain leucine-rich repeat protein phosphatase
PIP2	Phosphatidylinositol-4,5 bisphosphate

PIP3	Phosphatidylinositol 3,4,5-trisphosphate
PI3K	Phosphatidylinositol 3-kinase
PKB	Protein Kinase B
PP2A	Protein phosphatase 2A
P-REX1	Phosphatidylinositol 3-kinase-dependent Rac exchange factor
PTEN	Phosphatase and tensin homolog located at chromosome 10
P110β	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit beta isoform
RT-PCR	Real-time polymerase chain reaction
Ser	Serine
SNP	Single-nucleotide polymorphism
S6K1	Ribosomal protein S6 kinase beta-1
TCL1	T-cell leukemia/lymphoma protein 1
TGFβ	Transforming growth factor beta
Thr	Threonine

1 INTRODUCTION

1.1 CANCER: A GENERAL VIEW

"You have been diagnosed with cancer", is a painful statement and perhaps the worst medical news that in 2014 more than 14 million people worldwide will receive (GLOBOCAN 2012).

Cancer takes most life globally after cardiovascular diseases. There are geographic and sex diversity in both incidence and mortality of this disease. It is more common in developed countries and men are more susceptible to it. The American Cancer Society gives alarming facts for the coming future that: "Half of all men and one-third of all women in the US will develop cancer during their lifetimes" (Society 2014). There are several risk factors for cancer such as aging, but also to lifestyle (lack of physical activities, tobacco and alcohol consumption), environmental pollutions, chemical exposures, infections and associations with sex hormones (Anderson 2005).

Although cancer is a major health problem and its occurrence will unavoidably increase with time because of the growing and aging population, there is a lack of knowledge and common misconceptions amongst the public. The main reason for this is due the terminology of the word *cancer*, which is centuries old and has no scientific relevance in the nomenclature of the disorder itself. To name all cancers which rise from e.g. prostate for *prostate cancer* may give a false reflection to the nature of this complex and diverse disease.

Cancer is a general name which includes more than hundreds of types and many more subtypes. All of these subtypes differ in appearance, nature or symptoms that require distinct cares and treatments. No part of the human body is excluded from occurrence of cancer. Based on its local origin cancer has many subdivisions: *Sarcoma* is cancer originated in muscle, fibrous tissue, fat or bone and cartilage. *Leukemia* is cancer of blood cells arising in blood forming organs, bone marrow or spleen. *Lymphomas* affect the lymphatic system. *Carcinoma* (around 80% of all cancer) arises from the epithelium in organs, e.g. breast,

prostate, colon etc. Other types of cancer include melanoma and certain types of brain tumor.

Fortunately, nowadays the scientific community slowly shifts to rename different types of cancer based of their mutation pattern. That is only after increase use of personalized medicine.

Scientists propose different underlying mechanisms of the common character of cancer phenotypes. One of the most accepted characterization is described by *Hanahan D and Weinberg RA*. In 2000 they proposed six "hallmarks of cancer". They write: "six essential alterations in cell physiology that collectively dictate malignant growth: self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis" (Hanahan and Weinberg 2000). These hallmarks have been accepted widely, but also criticized lately since five of those hallmarks are a feature of benign tumors as well (except tissue invasion and metastasis) (Lazebnik 2010). Take this view into account, the scientific community still does not share a common theoretical position about some fundamental concepts and underlying principles of what makes cancer cancer. Questions such as: *when do the cell "decide" to metastases?* is still unanswered. Some argues that after decades of research and millions of papers we have still not been able to understand all the mechanisms which occur in normal cells, let alone cancer cells (Hanahan and Weinberg 2011). This brings us to a question of how we should design our future experiments to tackle this issue.

Direct comparison between normal and cancer cells, defining new behavior or unbalances and how mechanistic aspects differ, could be a good step forward. Not because we only find mechanisms which are changed or disturbed in cancer cells, but such contributions define opportunities to design new drugs which target those differences. Otherwise, we should be careful in drawing glorified conclusions which are based on single model studies. These events could lead us winning the battles, but losing the war.

1.2 PROSTATE CANCER

The exocrine prostate gland is a part of the male reproductive system. A normal prostate is about 3 cm long and localized in the pelvis, in front to the rectum, under the urinary bladder and encircling the urethra (Fig. 1). Mainly it produces and stores seminal fluid.

Male specific prostate cancer is the most common cancer in males in developed countries and the second most common worldwide (Figures 2008; GLOBOCAN 2012). Although prostate cancer is not among the highest lethal forms of cancer, it stands for second most cancer-related deaths in American men (Society 2014). The lower incidence of prostate cancer in developing countries may probably be due to the fact that diagnosing capability varies significantly. Studies on men above fifty years of ages have shown that incidentally undiagnosed prostate cancer is present in about 30–46% of all included subjects (Luczynska and Aniol 2013). Other investigations confirm similar observations when men who died of other reasons were studied. Prostate cancers were seen in 30% of all fifty years old men and 80% of seventy years old men (Breslow, Chan et al. 1977).

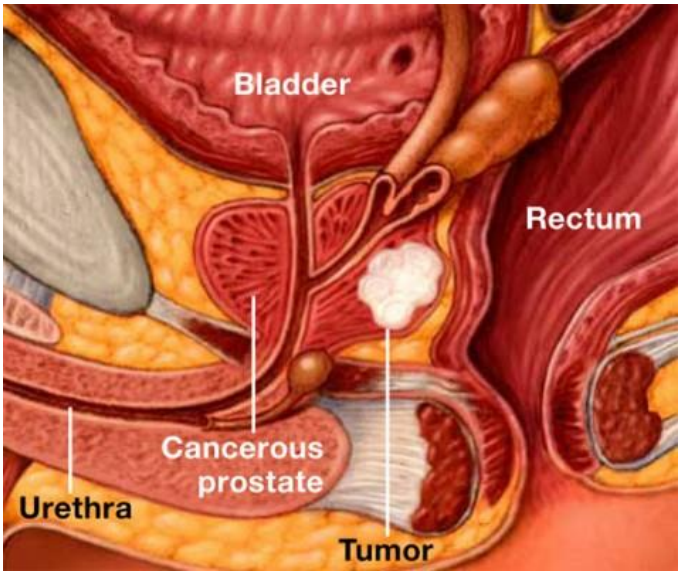

Figure 1: Location of the prostate and its cancer occurrence. *From reference* (Texas 2014).

The disease presents symptoms only when the tumor grows within the gland. Several symptoms have been reported, such as urinary, contains: transition from normal to blood colored, often and grievous urination, uncontrolled urination, insufficient sustain of a steady stream of the urine and inclusive chance of kidney failure. There are other late symptoms, such as drastic loss of weight (Institute 2014).

There are several risk factors of which the most prominent is age. Older men are much more susceptible, and its occurrence is rare in younger men (below 40 years). Genetic risk factor is obvious because some ethnicities are at higher risk, such as Afro-Americans. A familial pattern has also been reposted. Genes implicated or associated are PTEN, PHLPP, PIK3CA and others. (Torring, Borre et al. 2007; Taylor, Schultz et al. 2010). Obesity and smoking may also contribute to the occurrence of this disease.

1.3 THE GENETICS OF CANCER

Cancer development takes a long time, and several events are involved. Several attempts have been made in trying to classify cancer development (Farber 1984). Unlike other diseases, cancer is believed to be monoclonal and driven from a single cell. Based on this, the genetic alteration which occurs in a growing cell on its way toward malignancy is of high interest.

More than one single genetic alteration is needed for a cell to undergo malignant transformation. The process may start with a single mutation that leads to accumulation of further mutations. This affects the properties of the cell toward more genetic instability. Cancer development is a multistep process, and formation of a benign tumor is one of the earliest steps. Here, the cells do not respond to normal growth signals, but lack the capacity to invade other tissues. Not all benign tumors become malignant, but the chance to turn into malignancy increases for stepwise. In the case of colon cancer, genes have been shown to

become mutated in the sequence depicted in the Fig. 2 (Kinzler and Vogelstein 1996). The first mutation occurs in APS gene, which triggers progression of genetic alterations. Mutations in the P53 gene occur at later stages (Vogelstein and Kinzler 1993).

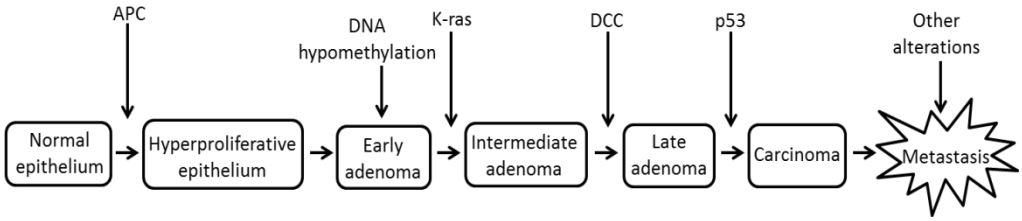

Figure 2: A possible genetic transformation cascade that leads to colon cancer development.

1.4 "MASTERS OF THEIR OWN DESTINIES"

Cancer is a disease on a cellular level. All cellular processes which occur in the normal body are tightly and carefully controlled. In the case of cancer, several phenotypic characteristics occur which are caused by genomic instability and they upset the balance of cellular processes. Based on these changes, cancer cells have been described as "masters of their own destinies" (Hanahan and Weinberg 2011). It takes a sufficient amount of time for a normal cell to evolve to become cancerous and important processes are involved. For instance, activation of oncogenes and suppression of tumor suppresser genes leads to evading growth suppression and escaping programmed cell death (Hanahan and Weinberg 2000).

However, cancer mortality is strongly dependent on other phenomena such as invasion, angiogenesis and metastasis. By largely unknown mechanisms, cancer cells, after very specific and self-selected moments, start to metastasize through the blood vessels and invade other tissues in the body (Hanahan and Weinberg 2011).

Yet another important feature of cancer cells is that they can proliferate unlimited and replicate in an infinite pattern. This growth factor independence is managed in different ways. Cells can produce their own growth factor ligands, they can affect surrounding non-cancer cells to provide them with the needed growth factors or deregulate receptor signaling by enhancing receptor proteins. Later, cancer cells can be proliferative signaling self-reliant also through constitutive activation/phosphorylation of some central cellular pathways which affect growth factor receptor/ligands. A pathway which frequently associates with hyper-activation or dysregulation in cancer is PI3K/Akt signaling pathway (Hanahan and Weinberg 2011). Dysregulation of this particular pathway has been reported in 42% of prostate cancer primary tumors and 100% of metastatic tumors (Taylor, Schultz et al. 2010). Disregulation is also common in other cancers e.g. brain, colon, breast, uterine and lung (Gao, Aksoy et al. 2013).

1.5 CELLULAR INVASION AND METASTASES

The majority of primary tumors cells, regardless of their origin, at some point during their development start to invade surrounding tissues. Cancer metastasis causes the vast majority of cancer related deaths (Sporn 1996). Metastasis and invasion are very complex processes and their mechanism remains poorly understood. Levels of proteins involved in cell to cell interactions and the extracellular matrix are altered in invasive cells. Extracellular proteases have been recognized as a general marker for invasiveness. Matrix metalloprotease family (MMP) is involved in the breakdown of the extracellular matrix and have been associated with cancer metastasis (Zhang, Hong et al. 2005; Rong, Li et al. 2013). Elevation of MMP9 expression has been shown to be due activation of PI3K/Akt pathway in prostate cancer cells (Dilly, Ekambaram et al. 2013).

1.6 AKT SIGNALING PATHWAY

The PI3K/Akt signaling pathway is frequently up-regulated in human cancers (Fresno Vara, Casado et al. 2004). Akt (also known as PKB) is an important molecule in cellular survival pathways. Akt is able to induce protein synthesis pathways, and is therefore a key protein involved in general tissue growth. Akt is

located in the cytoplasm and in the nucleus (Rosner and Hengstschlager 2012). Akt is over expressed in many tumors and by e.g. blocking apoptosis it might promote tumor cell survival. Akt is also involved in processes like DNA repair, metabolism, invasion and angiogenesis (Fig. 3) (Fresno Vara, Casado et al. 2004). The link between Akt pathway and cancer makes this pathway interesting not only in tumor development but also in cancer treatment.

The Akt gene was identified decades ago when a research group observed an occurrence of thymic lymphomas in their mouse bred. The Ak in the name Akt is from the mouse breed name and "t" stands for thymoma. Acute transforming retrovirus, which was called Akt8, was isolated from the Ak mouse strain. Because of the retrovirus, the oncogene was called v-Akt, then Akt when it was in human analogues (Staal, Hartley et al. 1977).

Later, several research groups worked in identification and characterization of Akt kinases. V-Akt was found to be a gene which is transduced by AKT8 retrovirus in rodents (Bellacosa, Testa et al. 1991), and later shown that in the cytoplasm of mouse cells, c-Akt (cellular homolog) encodes serine and threonine protein kinase Akt (Bellacosa, Franke et al. 1993). Kinases related to protein kinase A and C have been a focus of research and some researchers have been able to identity Akt, but call it protein kinase B (Coffer and Woodgett 1991; Jones, Jakubowicz et al. 1991).

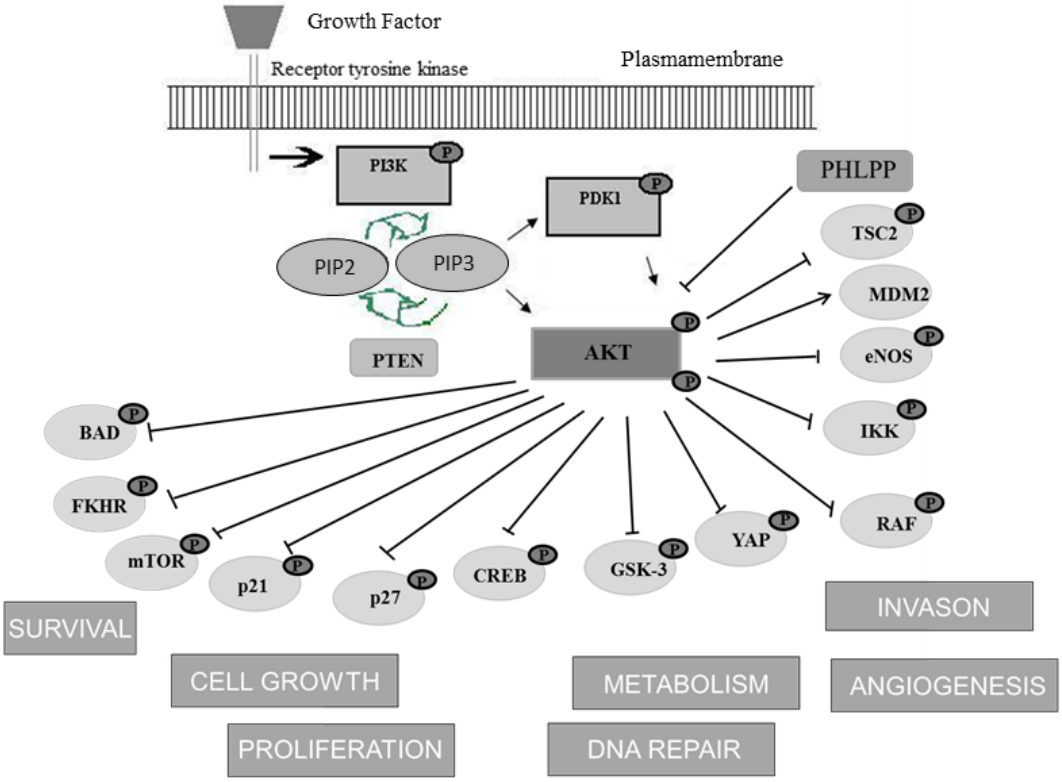

Figure 3: Schematic illustration of the Akt signaling pathway.

1.7 AKT ISOFORMS

There are three different isoforms of Akt: Akt1, Akt2, Akt3 (PKBα, PKBβ, PKBγ), and they are encoded by different genes. There are similarities in the structure of these isoforms. Akt family proteins contain a central kinase domain with specificity for serine or threonine residues in substrate proteins. In addition, the amino terminus of Akt includes a pleckstrin homology (PH) domain, which mediates lipid–protein and/or protein–protein interactions. The Akt carboxyl terminus includes a hydrophobic and proline-rich domain. Alignment of Akt family members suggests that the primary structure of Akt is conserved across evolution, with the exception of the carboxy-terminal tail, which is found in some but not all species and isoforms (Chan, Rittenhouse et al. 1999). Akt isoforms are expressed differently: AKT1 has the highest level of expression,

and is found everywhere (Bellacosa, Testa et al. 1991; Coffer and Woodgett 1991; Jones, Jakubowicz et al. 1991). Akt2 is widely expressed, but is mainly concentrated to skeletal muscle, adaptive tissue and liver (insulin sensitive tissues) (Jones, Jakubowicz et al. 1991; Konishi, Shinomura et al. 1994). Akt2 levels are massively enhanced during differentiation of some tissues (Hill, Clark et al. 1999; Vandromme, Rochat et al. 2001). Akt3, in comparison to the other two isoforms, is more organ specific, and is believed to be expressed mainly in brain and testis (Nakatani, Sakaue et al. 1999). Lately, a new role for Akt3 in aggressive breast cancer has also been reported (Chin, Yoshida et al. 2014).

1.8 ONCOGENIC AKT-ACTIVATION

Phosphatidylinositol 3-kinase (PI3K) is an Akt upstream/activator. PI3K synthesizes the important phosphatidylinositol 3,4,5-trisphosphate (PIP3) that binds to Akt. PIP3 binds directly to Akt´s pleckstrin homology domain. This binding leads to plasma membrane recruiting of Akt, wherein Akt kinase is phosphorylated and activated (Scheid and Woodgett 2003). This membrane recruitment phenomenon is not isoform specific but universal for all three Akt isoforms. From this perspective, the activity and amount of PI3K and PIP3 is absolutely crucial for Akt activity.

The phosphorylation sites between Akt isoforms do not vary much. Akt1 phosphorylates on Threonine (Thr) 308 or/and Serine (Ser) 473, Akt2 (Thr 309/Ser 474) and Akt3 (Thr 305/Ser472) (Hanada, Feng et al. 2004).

The Akt phosphorylation residues (Thr and Ser) are targeted by different kinases. Phosphoinositide-dependent protein kinase 1 (PDK1) phosphorylates Akt at Thr residues through direct binding between its PH domain and PIP3 (Bayascas 2010). While mammalian target of rapamycin complex 2 (mTORC2) phosphorylates Akt at Ser residue (Zoncu, Efeyan et al. 2011). There are other kinases which can phosphorylate Akt at Ser residues such as integrin-linked kinase (ILK) (Persad, Attwell et al. 2000). When Akt is phosphorylated in both sides it is fully activated. But it is emphasized that Thr is the more crucial

phosphorylation residue in an Akt activation point of view. Thr can alone phosphorylate several of Akt substrates, even when Ser phosphorylation is lacked (Guertin, Stevens et al. 2006).

1.9 NUCLEAR AKT

Akt has been found all over the cell. Activated/phosphorylated Akt translocates to different organelles such as Golgi, mitochondria, endoplasmic reticulum and nucleus. There are a variety of roles in these organelles such as diverse substrate phosphorylation or interplay with other cellular components (Martelli, Tabellini et al. 2012). The role of nuclear Akt has been highlighted lately and its presence has been emphasized as a key event in regulation of Akt signaling pathway. Nuclear Akt has been shown to be involved in processes such as cell cycle progression (Mistafa, Ghalali et al. 2010; van Opstal, Bijvelt et al. 2012), cell survival (Martelli, Tabellini et al. 2012), cell differentiation (Valverde, Benito et al. 2005), mRNA export (Okada, Jang et al. 2008), DNA repair (Bozulic and Hemmings 2009) and tumorigenesis (Vasko, Saji et al. 2004; Van de Sande, Roskams et al. 2005).

Some of the substrates of Akt are resident in the nucleus resident, such as transcriptional factor FOXO and p300 (Arden and Biggs 2002; Huang and Chen 2005). Furthermore, all Akt isoforms have been shown to be in or translocate to the nucleus, in the presence of several different stimuli. However the precise mechanism behind Akt nuclear localization/shuttling is still not known (Martelli, Tabellini et al. 2012). However, the role of a family of protein, proto-oncogene T-cell leukemia-1 (TCL1) has been suggested (Kunstle, Laine et al. 2002). Correlation between TCL1/Akt (PH domain) binding and Akt nuclear localization has been seen, but this observation needs to be studied more in detail, and it is not believed to be universal since the level of TCL1 is very low in variety of cellular models. Several fundamental questions are still debated about Akt localization. It is unclear if Akt has to be phosphorylated or not when it shuttles to the nucleus. It has been shown that when cells were transfected with

phosphorylated mutated Akt plasmids, Akt was observed in the nucleus, thus indicating that Akt localization is not dependent on its phosphorylation status (Saji, Vasko et al. 2005). Alternatively, overexpression of phosphorylation mutated Akt reduce nuclear Akt, even after growth factor treatment, and the authors claims that the phosphorylation of Akt is essential for its nuclear localization and activity (Xuan Nguyen, Choi et al. 2006). These contradictions might be due to differences in cell models and type of stimuli.

Noteworthy studies about different factors upstream of Akt have shown that the nucleus comprises all the required components to phosphorylate Akt, such as PI3K (Neri, Martelli et al. 1999), PIP3 (Neri, Bortul et al. 2002), mTORC2 (Rosner and Hengstschlager 2012) and PDK1 (Kikani, Dong et al. 2005). Finally, there are studies which claim that Akt is first localized to the nucleus after its phosphorylation in plasma membrane (Andjelkovic, Alessi et al. 1997; Ananthanarayanan, Ni et al. 2005), but other studies question these statements (Rubio, Avitabile et al. 2009).

1.10 PTEN

Phosphatase and tensin homolog (PTEN) is a well-known tumor suppressor and one of the major regulators of Akt. PTEN is mutated in both primary and more frequently in advanced stages of human cancers, including brain, breast, glioblastomas and prostate cancer (Ali, Schriml et al. 1999). PTEN is heavily associated with prostate cancer; in metastatic stage up to 70% of disturbed PTEN function have been reported (Taylor, Schultz et al. 2010).

PTEN is a dual function lipid protein phosphatase and inhibits the phosphorylation of Akt. PTEN converts PIP3 to phosphatidylinositol-4,5 bisphosphate (PIP2), thereby directly antagonizing the activity of PI3K (Fig. 4A) (Trotman, Wang et al. 2007). This function in particular makes PTEN the key tumor suppressor. PTEN loss is correlated to activation of Akt pathway and PIP3 plasma recruitment which in turn drives the cell into multiple processes, for instance enhance proliferation, avoiding apoptosis.

The size of human PTEN is 403 amino acids which are divided in two bigger domains: a phosphatase and a C2 domain, followed by three shorter regions: N-terminal (responsible for its PIP2 binding capacity), PEST sequences comprised of a C-terminal tail and a PDZ interaction motif (Fig. 4B.) (Lee, Yang et al. 1999). The phosphatase activity of PTEN is known to be dependent on its N-terminal phosphatase domain, but in contrast to that the significance of the C terminal has also been discussed. Note also that 40% of the PTEN mutations are in the C-terminal C2 domain and its tail sequence (Waite and Eng 2002). Available reports stresses that C- terminus is crucial for PTENs binding capacity for many proteins (Fan, He et al. 2009), membrane recruitment and phosphatase activity (Odriozola, Singh et al. 2007).

In non-malignant tissue, PTEN is constantly expressed, and transcriptional and epigenetic regulators have been proposed to regulate PTEN level, e.g. transcriptionally: PTEN mRNA, TGF-β (transforming growth factor β) (Li and Sun 1997), post-transcriptionally: miR21, miR26a and miR214 (Meng, Henson et al. 2007; Yang, Kong et al. 2008; Liu, Wu et al. 2012).

Several repports show that PTEN shuttles to the nucleus and regulates PIP3 levels and hence Akt activity. PTEN does not contain nuclear import or export signals and several mechanisms have been suggested for nucleo-cytoplasmic shuttling of PTEN (Trotman, Wang et al. 2007). It is confirmed that mono-ubiquitination of PTEN is critical for its nuclear localization. In a similar vein, E3 ubiquitin ligase NEDD4-1 (neural precursor expressed developmentally down-regulated 4-1), which is HECT domain protein, has been pointed out to be a determing factor in PTEN activity and nuclear localization through catalyzing the mono-ubiquitination process (Trotman, Wang et al. 2007; Wang, Trotman et al. 2007; Fouladkou, Landry et al. 2008). An indirect effect of NEDD4-1 mediated PTEN mono-ubiquitination is that cytoplasmic PTENs both poly-ubiquitination and degradation are blocked. In addition, other mechanism behind nuclear shuttling of PTEN cannot be excluded. The relevance of both passive transport by diffusion (Liu, Wagner et al. 2005) and NLS-mediated (nuclear localization signal) active transport (Chung and Eng 2005) have been

highlighted. Finally, the nuclear PTEN seems important for cancer suppression through inhibition of phosphorylated Akt.

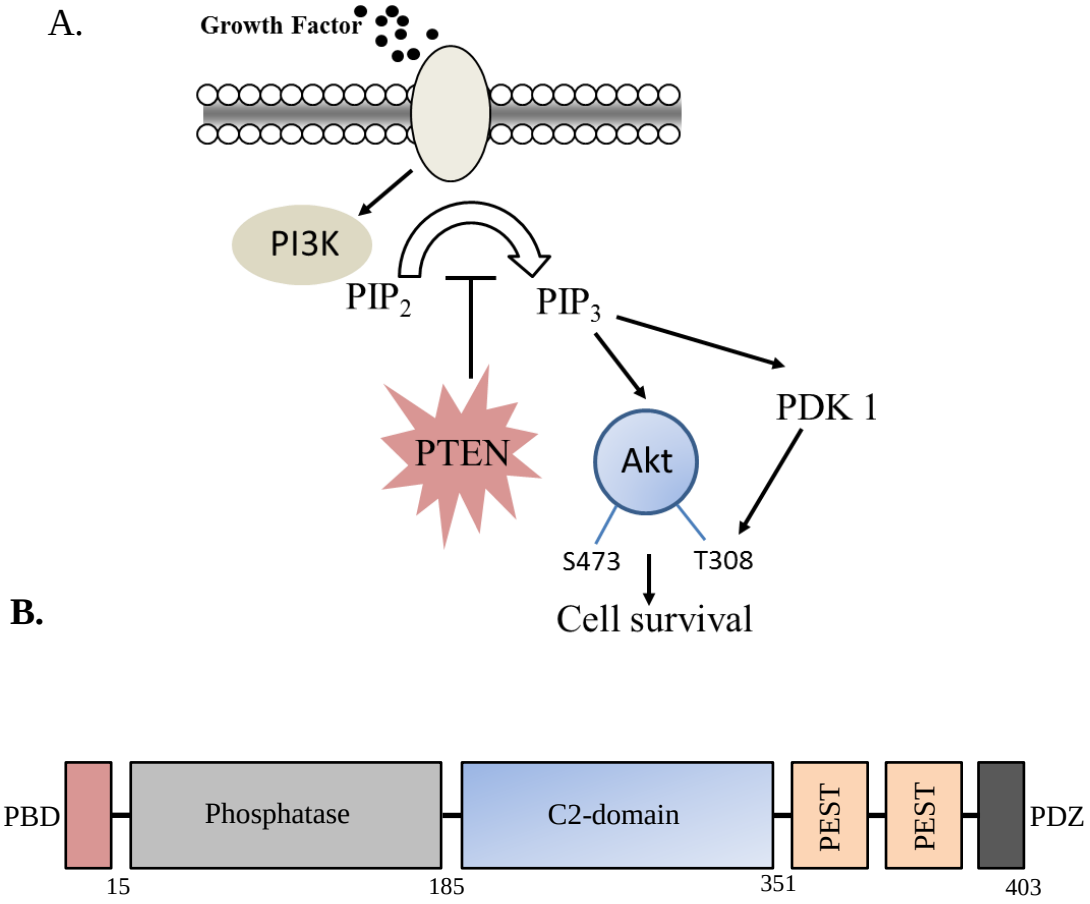

Figure 4: A, Schematic illustration of PTEN regulation. **B**, structure of PTEN.

1.11 PHLPP

Even though phosphorylation at Thr is identified to be the crucial one, the Ser phosphorylation massively enhances Akt activity, controls the remaining of the Akt activation state and continual phosphorylation of Thr site (Yang, Cron et al. 2002). For that reason, Ser phosphorylation is presumably the determining factor in the impact and continuance of Akt activity. Akt is dephosphorylated on Ser residue primarily by a recently discovered phosphatase, PH domain leucine-rich repeat protein phosphatase (PHLPP) (Brognard, Sierecki et al. 2007).

PHLPP directly dephosphorylates the hydrophobic motif of Akt, resulting in inhibition of kinase activity and promotion of apoptosis (Brognard and Newton 2008; Gao, Brognard et al. 2008). There are two genes which encode this Ser specific PHLPP phosphatase: PHLPP1 and PHLPP2. PHLPP1 further has two major splice variants, PHLPP1α and PHLPP1β.

PHLPP1 and PHLPP2 isoforms have been shown to dephosphorylate and therefore acutely inactivate different Akt isoforms. PHLPP1 is specific for Akt2 and Akt3 whereas PHLPP2 dephosphorylates Akt1 and Akt3 (Fig. 5) (Brognard, Sierecki et al. 2007). It has been shown that both isoforms are present in cytosolic, nuclear and membrane fractions. Beside of PHLPPs direct effect on Akt, an indirect effect (feedback loop) of PHLPP has been discussed. It has been shown that PHLPP directly dephosphorylates an Akt downstream S6K1. Furthermore, it has been shown that loss of PHLPP leads to an activation of S6K1 (Liu, Stevens et al. 2011). PHLPP activity has been correlated with its binding to scaffolding proteins, such as FKBP51, but this observation is believed to be cell specific (Pei, Li et al. 2009). PHLPP affects other substrates of Akt, e.g. MST1, a proapoptotic kinase which is catalyzed by Akt through phosphorylation. PHLPP binds to MST1 and dephosphorylates it, resulting in facilitation of apoptosis (Qiao, Wang et al. 2010). Not much is known about regulation of PHLPP but recently an epigenetic regulator, miR190, has been discussed (Beezhold, Liu et al. 2011).

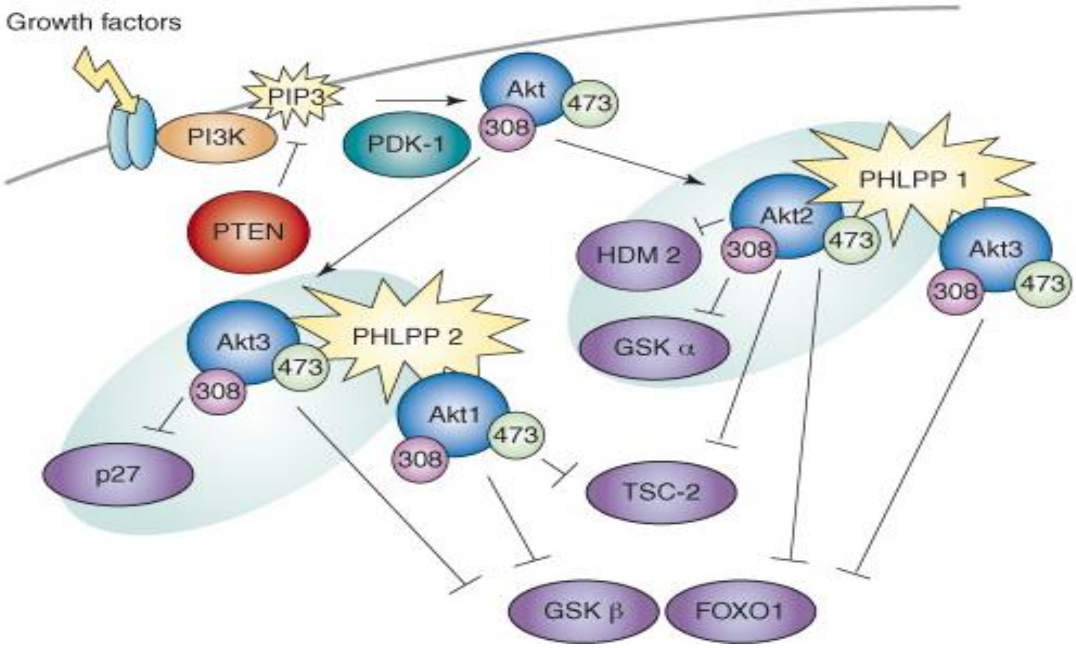

Figure 5: Schematic illustration of PHLPP regulation. Figure from (Brognard and Newton 2008) with permission from the author.

1.11.1 PHLPP and PTEN interplay in cancer

PTEN has an established role in prostate cancer and there is a solid ground of data which highlights its involvement. As emphasized, PTEN and PHLPP affect phosphorylated Akt in different ways. PTEN prevent phosphorylation of Akt while PHLPP dephosphorylates Akt. PHLPP is not as well studied, but new genetic discoveries illustrate genetic deletion in both PHLPP1 and PHLPP2, to the same degree as PTEN in prostate cancer (Taylor, Schultz et al. 2010). The role of PHLPP1 in prostate cancer was further supported when PHLPP1 knockout mice were studied (Chen, Pratt et al. 2011). Interestingly, the disease process was faster because these PHLPP1 knockout mice got deletions in the PTEN gene. Surprisingly, elevated PHLPP2 levels were seen, and that slowed down the rate of the disorder, but did not prevent it. Later, interplay between

PTEN and PHLPP was demonstrated in both human and mouse cells. Cells with PHLPP1 and PTEN deletion had an elevated PHLPP2 (Chen, Pratt et al. 2011). A new line of inquiry showed cross-talk between PI3K and androgen receptor pathway in mouse prostate PTEN null cells. This resulted in PHLPP down regulation and increased proliferative capacity through Akt pathway. (Mulholland, Tran et al. 2011).

There are very little mechanistic studies on PHLPP and PTEN interplay, but scaffolding NHERF (Na^+/H^+ exchanger regulatory factor 1) has been shown to bind to both PTEN and PHLPP, and affect patient survival through Akt pathway. (Molina, Agarwal et al. 2012). Newton and Trotman writes in their latest review about this interplay: "Multiple lines of evidence have confirmed that a hierarchical organization for compensation among PTEN, PHLPP1, and PHLPP2 exists" (Newton and Trotman 2014). It remains to see how this phenomenon affects tumor development. Surely, in coming years this will be characterized by new mechanistic studies.

1.12 PI3K SUBUNITS (P110B)

PI3K are heterodimeric molecules consisting of a catalytic subunits (p110), and regulatory subunits (p85) (Liu and Roberts 2006). There are at least three P110 subunits: P110α P110β and P110δ. Different growth factor stimulation leads to catalyzing PI3K by P110 to the membrane. *In vitro*, P110β and δ have been shown to mediate PI3K activation in PTEN-deficient prostate cells (Jiang, Chen et al. 2010). Furthermore, it acts as an oncogene and is critical for prostate tumor development in PTEN knockout mice (Jia, Liu et al. 2008). Expression of p110β has been noticed to increase with aggressiveness in prostate cancer. Nuclear pAkt, which is involved in prostate tumorigenesis, is regulated by p110β. Binding between nuclear p110β and nuclear Akt has also been seen. It is even shown that PTEN-null tumors are sensitive to P110β isoform inhibitors (Torbett, Luna-Moran et al. 2008).

1.13 EHBP1 AND P-REX1

Evidence indicates that activation of PI3K and its downstream targets are required for insulin-induced transport processes, through promotion of GLUT4 (a glucose transporter) to translocate at the plasma membrane (Ros-Baro, Lopez-Iglesias et al. 2001), and depletion of PI3K or Akt2 inhibiting insulin signaling (Clarke, Young et al. 1994; Jiang, Zhou et al. 2003). Recent, findings argue for a crucial role of EH the domain-binding protein 1 (EHBP1) in the insulin-mediated rapid receptor trafficking and other translocations (Guilherme, Soriano et al. 2004; Bravo-Cordero, Marrero-Diaz et al. 2007; Jovic, Naslavsky et al. 2007; Shi, Chen et al. 2010). In addition, in genome-wide association studies, associations between EHBP1 and aggressive prostate cancer have been highlighted (Gudmundsson, Sulem et al. 2008; Waters, Le Marchand et al. 2009). It is worth mentioning that very little is known about cellular functions of EHBP1, even though some observations indicate its clear involvement in aggressive prostate cancer.

Like EHBP1, P-Rex1 (PI3K-dependent Rac exchange factor) which is a guanine nucleotide exchange factor, has been shown to affect the rapid translocation of GLUT4, and thereby insulin trafficking in a PI3K-dependent manner (Balamatsias, Kong et al. 2011). In prostate cancer cells, a critical role of P-Rex1 in invasive growth is ascribed. The level of P-Rex1 is correlated with cellular invasiveness (Qin, Xie et al. 2009). Furthermore co-localization between PTEN and P-Rex1 has been demonstrated (Dillon 2013).

PI3K dependent transporter proteins which are associated to rapid cellular effects are interesting for increased understanding for the mechanism behind rapid nuclear Akt depletion, which makes both EHBP1 and P-Rex1 good candidates for such investigation.

1.14 P2 RECEPTORS

There is complex cellular communication between and/or within the cells. The role of nucleotides as an extracellular messenger has been brought to light. Varieties of cells, after exposing for precise stimulation, release P2 receptors, which are a class of plasma membrane receptor (Abbracchio and Burnstock 1994; Muller 2002). Based on their structure, transduction features and pharmacological characteristic, two groups of P2 receptor have been classified: P2Y and P2C. P2 receptors are stimulated by nucleotides and their natural ligand is ATP and its metabolites (Shabbir and Burnstock 2009) (Fig. 6).

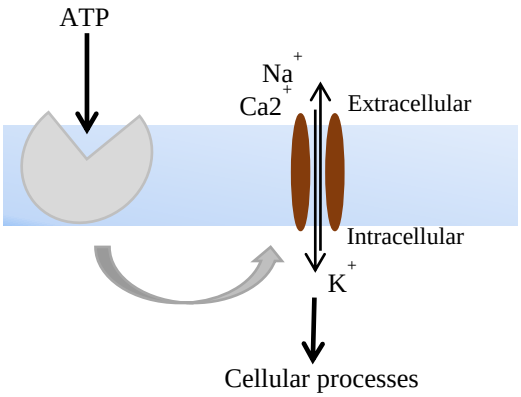

Figure 6: The predicted structure of the P2XR receptor. ATP is the physiological ligand.

1.14.1 P2X receptors

P2X receptors are cation-selective (Na^+, K^+ and Ca^{2+}) and ligand-gated ion channels. These ATP-gated plasma membrane channels have seven different subunits: P2X1-7 (Di Virgilio 2012). Rapidity is a characteristic of P2X, stimulation results to permeability of cations within 10ms (Shabbir and Burnstock 2009). The structures of human P2X are quite similar except the P2X7 subunit, which is longer. For instance P2X4 is 388 amino acids long, while P2X7 is 588(Khakh and North 2006), with a carboxyl-terminal tail of 242 residues (Surprenant, Rassendren et al. 1996). Changes in the intracellular ion

concentration are classified to the primary signal-transduction mechanism for all P2Xs, but other roles of P2X7 have been reported, such as interaction with at least 11 different proteins which are involved in several cellular endpoints (Kim, Jiang et al. 2001).

1.14.2 P2X7 receptor

The P2X7 was discovered for the first time in mammalian sensory neurons, but nowadays shown to be expressed in several cell types e.g. endothelial cells, smooth muscle cells, immune cells, macrophages, dendritic, fibroblasts and lymphocytes (Di Virgilio, Chiozzi et al. 2001; Adinolfi, Pizzirani et al. 2005). Elevations of P2X7 were observed in chronic pancreatic and pancreas cancer, suggesting a role of P2X7 in the pancreas cancer development (Kunzli, Berberat et al. 2007).

Activation of P2X7 has been shown to mediate apoptosis through effects on estrogen (Wang, Wang et al. 2004). P2X7 effects are reported in both mitochondrial – caspase-9-induced (Feng, Wang et al. 2005) and ATP mediated apoptosis (Coutinho-Silva, Persechini et al. 1999). Agonists of P2X7 induce nuclear localization of various transcription factors for example NFAT and NFκB (Ferrari, Wesselborg et al. 1997; Ferrari, Stroh et al. 1999).

P2X7 has been called *the suicide receptor*, since prolonged agonist exposure leads to cytotoxic pore-forming resulting in apoptosis or necrosis (Shabbir and Burnstock 2009; Di Virgilio 2012).

Correlations between P2X7 and cancer have been subject for research and P2X7s involvement in tumor growth is now unquestionable. Some studies state that most malignant tumors overexpress P2X7 (Di Virgilio, Ferrari et al. 2009). Functionality of P2X7 has been connected to the invasive capacity of cancer cells, and is believed to be a crucial parameter in the development of metastases (Jelassi, Chantome et al. 2011). Altered expression of P2X7 is seen in patients with prostate cancer compared with those who had normal prostates. The majority of early biopsies from prostate cancer were stained positively for P2X7 (114 of 116 biopsies), and at later stage non-functional P2X7 were founded in all

116 pathological samples. (Slater, Danieletto et al. 2004). This enhanced level of P2X7 in tumors is speculated to be a step forwards activation of apoptosis in cancer cells, but apoptosis fails since the receptor is dysfunctional (Slater, Barden et al. 2000).

1.14.3 P2X4

P2X4 is not as well studied as P2X7, but except in nervous tissue it is known that P2X4 is expressed in epithelial and endothelial tissue (Khakh and North 2006). Some of the most interesting functions of P2X4 are its implication in wound healing (Freeman, Bowman et al. 2011) and neuropathic pain. P2X4-positive microglia has been suggested as an target for chronic pain treatment (Tsuda, Masuda et al. 2013). Nerve injury transforms microglia cells so that they overexpress and re-distribute P2X4 to the plasma membrane (Beggs, Trang et al. 2012). It is shown that P2X4 and P2X7 interact and bind each other (Craigie, Birch et al. 2013; Hung, Choi et al. 2013).

1.15 STATINS

Cholesterol, which is a cornerstone for mammalian cell membrane structure and function, has been implicated in heart disease. Statins or HMG-CoA reductase inhibitors are a category of drugs that prevent cardiovascular disease and that act by inhibiting the rate limiting step the in mevalonate pathway synthesizing cholesterol (Bellosta, Paoletti et al. 2004; Raval, Hunter et al. 2011). Blocking the mevalonate pathway affects a number cellular functions, because e.g. prenylation, which is a result of mevalonate pathway, has been shown to be essential for activation of several proteins including some oncogenes.

Beside its role in cardiovascular disease prevention, it has been emphasized that statins may prevent cancer (Wong, Dimitroulakos et al. 2002; Graaf, Beiderbeck et al. 2004; Graaf, Richel et al. 2004; Jacobs, Rodriguez et al. 2007). Use of statins (> 4 years) has been associated with a spectacular (< 50%) risk reduction

in prostate cancer (metastatic or fetal), and 80% lower risk for pancreatic cancer (Platz, Leitzmann et al. 2006; Khurana, Sheth et al. 2007). Beside effects on cancer risk it has been shown that statins have antitumor effects *in vivo*. It has been demonstrated that statins increase the efficacy of diverse anticancer drugs in different animal models (Broitman, Wilkinson et al. 1996; Hawk, Cesen et al. 1996; Narisawa, Fukaura et al. 1996; Inano, Suzuki et al. 1997; Feleszko, Mlynarczuk et al. 2002; Kusama, Mukai et al. 2002). In humans, statins have also been used in combination with cytostatic drugs (Kornblau, Banker et al. 2007; Schmidmaier, Baumann et al. 2007) and effects on median survival have been seen (Kawata, Yamasaki et al. 2001).

In vitro, several cellular effects have been reported e.g. reduction of invasive and proliferative capacity, activation of apoptotic signals and radio-sensitizing effects (Hoque, Chen et al. 2008; Oliveira, Zecchin et al. 2008; Brown, Hart et al. 2012; He, Mangala et al. 2012). Recently, several reports indicate that statins may act through the Akt signaling pathway. (Mistafa, Hogberg et al. 2008; Mistafa and Stenius 2009; Chen, Lan et al. 2012; Wu, Yang et al. 2013).

Different mechanisms have been suggested, such as Ras prenylation (Peres, Yart et al. 2003; Graaf, Richel et al. 2004) or effects on phosphorylated nuclear Akt depletion (Roudier, Mistafa et al. 2006), and P2X7 receptor has been suggested to be a key mediator of this effect (Mistafa, Hogberg et al. 2008; Mistafa and Stenius 2009). Finally, statins present as a good Akt inhibitor and this observation gives a solid ground that makes statins a suitable drug to use for manipulating the Akt pathway.

2 AIM OF THE STUDY

The general aim of the thesis is to increase understanding about:

- How the Akt pathway is regulated.
    - Focus on the role of receptors and different phosphatases.
- Potential effects of this pathway
- Mechanism of action

The specific aims were as follow:

The aim was to study the mechanism behind pAkt nuclear depletion (**paper I**), identify the involvement of crucial proteins (**paper II**), later determine the role of Akt phosphatases in drug response (**paper III**), and finally, study the relation between these phosphatases in detail (**paper IV**).

3 MATERIAL AND METHODS

To find a more precise and detailed description, see the specific paper (paper I-IV). Below is a brief description of the main used material and methods.

3.1 CELL CULTURE (PAPER I-IV)

Several different cell lines were used. In paper I, Non-small cell lung cancer cells (A549) and human prostate carcinoma cell line (LNCaP) were used. In paper II, mouse embryonic fibroblasts p110β-null, p110β-wild type, A549, prostate cancer DU145 cells, and pancreatic cancer Panc-1 cells were used. In paper III and paper IV, the human prostate carcinoma cell lines DU145, 22RV1, LNCaP, PC3 and immortalized prostate luminal epithelial (non-tumorigenic) RWPE-1 cells were used. In paper IV, human breast adenocancinoma MCF7 cells, human prostate stem cells WPE, Mouse embryonic fibroblasts (MEFs), non-tumorigenic rat liver TRL1215 cell line, Human embryonic kidney (HEK) 293 cells stably expressing human empty vector, P2X4 or P2X7 were also used.

DU145, 22RV1, LNCaP, PC3, WPE, RWPE-1, A549, Panc-1 and MCF7 cells were purchased from American Type Culture Collection, ATCC (Manassas, VA). p110β-null and p110β-wild type (MEFs), were kindly provided by Dr. Jing Zhang (Harvard Medical School, Boston). TRL 1215 cells were provided as a generous gift from Dr. Michael P. Waalkes from the National Cancer Institute. This non-tumorigenic cell line was originally derived from the livers of 10-day-old Fischer F344 rats (13). Human embryonic kidney (HEK) 293 cells stably expressing human P2X4 and P2X7 were kindly provided by A. Surprenant, Sheffield University, UK. Human embryonic kidney (HEK) 293 cells stably expressing human empty vector were kindly provided by Prof. A. North, University of Manchester, UK.

DU145, A549, Panc1, MCF7 and MEF cells were grown in Dulbecco's modified Eagle medium, D-MEM, with 10% inactivated fetal bovine serum (FBS), penicillin-streptomycine and 1 mM sodium pyruvate. 22RV1 cells were grown in RPMI-1640 supplemented with 10% inactivated FBS and penicillin-streptomycine. PC3 cells were grown in RPMI-1640 supplemented with 10% inactivated FBS, 1 mM sodium pyruvate, 2 mM L-Glutamine and penicillin-streptomycine. LNCaP cells were additionally supplemented with 1mM HEPES. The RWPE-1 and WPE cells were grown in kerantinocyte SFM (GIBCO 17005), with bovine pituitary extract, EFG human recombinant and antibiotic-antimycotic (GIBCO 15240). The culturing of WPE cells was according the procedure for ATCC CRL-2887. The TRL 1215 cells were grown in William´s E+GlutaMaxTM-I with penicillin/streptomycin and 10% inactivated FBS. HEK293 P2X4 and P2X7 cells were grown in DMEM:F12 with 1 mM l-glutamine, 10% inactivated FBS and 300 µg/ml G418. Human embryonic kidney (HEK) 293 control cells (overexpressing empty vector) were grown in DMEM:F12 (GIBCO 21331), 2 mM l-glutamine, 10% inactivated FBS.

3.2 PROTEIN ANALYSES (PAPER I-IV)

3.2.1 Western blotting (paper I-IV)

Western blotting is a semi-quantitative method and used for a detection of specified proteins. This method separates the proteins in the sample content based on their size. In brief, cells were lysed in IPB-7 buffer (Triethaolamine-HCL (TEA) 1M PH 7.8, NaCL 5M, sodiumdeoxycholate (DOC) 4%, Igepal CA-630 or NP-40 10%) with inhibitors (1 mg/ml PMSF, 0.1 mg/ml trypsin inhibitor, 1 mg/ml aproteinin, 1 mg/ml leupeptin, 1mg/ml pepstatin, 1 mM Na3VO4 and 1 mM NaF). The samples were subjected to SDS–PAGE and blotted onto a PVDF membrane (Bio-Rad, Hercules, CA). The protein bands were probed using antibodies (for specific antibodies see paper I-IV). Proteins were visualized with ECL procedure (Amersham Biosciences, Sweden). The

Western blotting results were analyzed with NIH Image 1.62 software.

3.2.2 Chromatin isolation (paper IV)

Chromatin was isolated essentially as described in (14). Cells were lysed in IPB-7. Two fractions were isolated by centrifugation (14,100 x g): the supernatant containing the cytoplasm and the soluble nuclear fraction and nonsoluble pellet containing the chromatin (14).

3.2.3 Immunoprecipitation (paper I, II and IV)

Immunoprecipitation was performed by using antibodies (for specific antibodies see paper I, II and IV) and protein A/G PLUS-agarose (Santa Cruz, CA). Cells were washed with PBS and lysed in IPB-7. The cell lysates were incubated for 1 h with antibodies and thereafter with protein A/G PLUS-agarose for 24 h at 4 °C.

3.3 TRANSFECTION METHODS

3.3.1 RNA interference

Cells were transfected with P2X4, P2X7, PTEN or control small interference RNA (siRNA) (Santa Cruz Biotechnology, Santa Cruz, CA) for 40 h or for times indicated in the figures according to the TranIT-TKO protocol (LipofectamineTM 2000, Invitrogene).

3.3.2 Inhibition of microRNA (paper IV)

Cells were transfected with anti-microRNA 190 (anti-miR190), anti-microRNA 214 (anti-miR214) and microRNA negative control (Non–targeting, NT) inhibitor (mirVanaTM miRNA inhibitors, Ambion life technologies, Bleiswijk, Netherlands) for 40 hours according to the mirVanaTM miRNA inhibitors, Ambion protocol. Cells were transfected using LipofectamineTM 2000 (Invitrogene) as transfection reagent.

3.3.3 Plasmid transfection (paper I and IV)

Cells were transfected with diverse plasmids (see paper II and IV) according to the LipofectamineTM 2000 (Invitrogene) protocol. Cells were transfected for 4µg plasmid per 60mm dish (or as indicated in the figures for paper IV). (according to the TranIT-TKO protocol) and for times indicated in the papers.

3.4 PCR ANALYSES

3.4.1 RNA Purification and Real-Time RT-PCR (paper IV)

Total RNA was prepared using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and cDNA was generated with the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) according to protocol. Subsequently, quantification of gene expression was performed in duplicates using Maxima™ SYBR® Green qPCR Master Mix (Fermentas, St. Leon-Rot, Germany) with detection on an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). The reaction cycles used were 95 °C for 2 min, and then 40 cycles at 95 °C for 15 s and 60 °C for 1 min followed by melt curve analysis. Primer sequences are given in paper IV. Relative gene expression quantification was based on the comparative threshold cycle method (2–ΔΔCt) with normalization of the raw data to the included housekeeping gene (GAPDH). Quantification of miR was performed using a miRCRY LNA Universal RT miR cDNA synthesis kit, SYBR Green master mix, Universal RT, and LNA PCR primer set for miR16, miR21, miR26a, miR107, miR190 and miR214, normalized to miR103 (Exiqon, Vedbaek, Denmark). Relative gene expression quantification was based on the comparative threshold cycle method ($2^{-\Delta\Delta Ct}$).

3.5 CELL VIABILITY AND INVASION ASSAY

3.5.1 MTT assay (paper II, III and IV)

Cell viability was determined by 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay detecting the cellular mitochondrial capacity to convert MTT tetrazolium salt to formazan. Cells were incubated with the medium containing MTT (Sigma–Aldrich, St. Louis, MO) for 4 h. The cells were then lyzed in DMSO. The absorbance was measured at 570–620 nm.

3.5.2 Cell Invasion assay (paper III and IV)

Cell invasion assay was performed using 8-μm pore size Transwell Biocoat Control inserts (Becton Dickinson, Bedford, MA) according to the manufacturer's instructions. The cells were fixed with methanol and thereafter stained with Toluidine Blue from Merck (Darmstadt, Germany). The number of transmembrane cells was counted.

3.6 *IN VIVO* ANALYSES

3.6.1 Animal Experiments (paper IV)

Female Sprague–Dawley rats were injected intraperitoneally with DEN (300 μmol/kg body weight) (Sigma–Aldrich, St. Louis, MO), dissolved in 0.15 M NaCl within 24 h after birth. At three weeks of age, these rats were weaned and injected thereafter with the same dose of DEN once every other week. After 11 additional doses visible hepatic lesions (preneoplastic tissue) and hepatic tissue without visible lesions (control tissue) were dissected out and homogenized in 0.25M sucrose. Samples from EAF and non-EAF tissue were analyzed by Western blotting. All experiments involving animals were approved by the local ethical committee according to the guidelines of the Swedish National Board of Laboratory Animals. Institutional guidelines for the proper, humane use of animals in research were followed.

3.7 MICROSCOPY

3.7.1 Immunocytochemical Staining (paper I, II and III)

Cells were fixed in 3.7% formaldehyde. After fixation, cells were stained with antibodies (for specific antibodies see paper I-III). After incubation with primary antibodies at 4 °C overnight, secondary antibody conjugated with FITC or Texas Red were applied (Dako, Glostrup, Denmark). No staining was detected when the primary antibodies were omitted. The staining intensity was analyzed with NIH Image 1.62 software.

3.7.2 Proximity Ligation Assay (paper I and III)

The proximity ligation assay (PLA) was performed according to the manufacturer's protocol using the Duolink detection kit with PLA PLUS and MINUS probes for mouse and rabbit (Olink Bioscience, Uppsala, Sweden).

3.7.3 Confocal microscopy (paper III)

Cells were fixed with 4% formaldehyde, permeabilized with 0.2% Triton X-100 in 2% bovine serum albumin buffer. Immunostainings were performed using antibodies (for specific antibodies see paper III). Secondary antibody conjugated with Alexa 488 (rabbit) and Alexa 594 (mouse). Samples were mounted in 4′,6-diamidino-2-phenylindole. Fixed cells were performed with a Zeiss LSM 510 META confocal laser scanning microscope (Zeiss, Oberkochen, Germany) equipped with ×63 Plan-Aoil-immersion lens. An agron laser was excited at 488 nm and fluorescence image was recorded from 500 to 550 nm. A helium–neon1 laser was used for excitation at 543 nm and emission from 560 to 615 nm. Colocalization of indicated proteins was measured by Zeiss LSM imaging software in multitrack mode.

3.8 GENETIC ANALYSES

3.8.1 Genetic analysis of P2X7 (paper III)

The publicly available American Cancer Genetic Markers of Susceptibility (CGEMS) study, comprising 1172 prostate cancer cases and 1098 controls, was used (Yeager, Orr et al. 2007). Among the patients, disease aggressiveness was defined by the CGEMS study as follows: patients with clinical stage T3/T4 or Gleason score of 7 or higher based on biopsy specimens were classified as having more aggressive disease, whereas the remaining patients were classified as having less aggressive disease. All participants gave written informed consent.

4 RESULTS AND DISCUSSION

4.1 PAPER I: PURINERGIC RECEPTOR-MEDIATED RAPID DEPLETION OF NUCLEAR PHOSPHORYLATED AKT DEPENDS ON PLECKSTRIN HOMOLOGY DOMAIN LEUCINE-RICH REPEAT PHOSPHATASE, CALCINEURIN, PROTEIN PHOSPHATASE 2A, AND PTEN PHOSPHATASES

Akt is an important oncoprotein, and data suggests a critical role for nuclear Akt in cancer development. The mechanism behind the rapid inhibition of nuclear pAkt induced by ATP and statins was studied. Members in our research group have previously described a rapid (3–5 min) and P2X7-dependent depletion of nuclear pAkt and effects on its downstream targets (Roudier, Mistafa et al. 2006; Mistafa, Hogberg et al. 2008), and here, the mechanism behind the pAkt depletion was studied.

Calcineurin has been shown to be activated by Ca^{2+} and its role in Akt dephosphorylation is documented (Park, Kim et al. 2008). Atorvastatin enhances Ca^{2+} level in epithelial cells (Mistafa, Hogberg et al. 2008). Protein phosphatase 2A (PP2A) dephophorylates Akt at both phosphorylation sites (Ser473 and Thr308), and is also regulated by Ca^{2+} (Ahn, Sung et al. 2007). PTEN is negative regulator of Akt (Rabinovsky, Pochanard et al. 2009) while PHLPP dephosphorylates Akt directly (Gao, Furnari et al. 2005).

We show that cholesterol-lowering drugs, statins, or extracellular ATP, induced a complex and coordinated response in insulin-stimulated A549 cells leading to depletion of nuclear pAkt. This involved protein/lipid phosphatases PTEN, PHLPP1 and -2, PP2A, and calcineurin.

We showed that PHLPP and calcineurin translocated to the nucleus and formed complexes with pAkt within 3 min. Also PTEN translocated to the nucleus and then co-localized with pAkt close to the nuclear membrane. FK506-binding protein 51 (FKBP51) has been shown to act as a scaffolding protein for Akt

and PHLPP and thereby regulate for PHLPP activity (Pei, Li et al. 2009). FKBP51 is shown to be regulated by calcineurin (Li, Baksh et al. 2002).

FK506, an inhibitor of FKBP51 and calcineurin, prevented depletion of nuclear pAkt. Furthermore, okadaic acid, an inhibitor of PP2A, prevented nuclear pAkt depletion. Chemical inhibition and siRNA indicated that PHLPP, PP2A, and PTEN were required for a robust depletion of nuclear pAkt, and in prostate cancer cells lacking PTEN, transfection of PTEN restored the statin-induced pAkt depletion.

We studied the pAkt nuclear depletion effect on other cellular endpoints, e.g. cell cycle progression. Proliferating cell nuclear antigen (PCNA) and $p21^{cip1}$ were investigated since they are nuclear targets of Akt and have effects on the cell cycle (Rossig, Jadidi et al. 2001). Nuclear PCNA is known to bind to PP2A (Morrow, Tung et al. 2004)

The overall activation of protein and lipid phosphatases was paralleled by a rapid PCNA translocation to the nucleus, a PCNA-$p21^{cip1}$ complex formation, and cyclin D1 degradation. We conclude that these effects reflect a signaling pathway for rapid depletion of pAkt that may stop the cell cycle.

Taken together, the findings in paper I show that cholesterol-lowering drugs, statins, or extracellular ATP, induce a complex and coordinated response in insulin-stimulated A549 cells leading to depletion of nuclear pAkt (Fig. 7). This involved protein/lipid phosphatases PTEN, PHLPP1 and -2, PP2A and calcineurin.

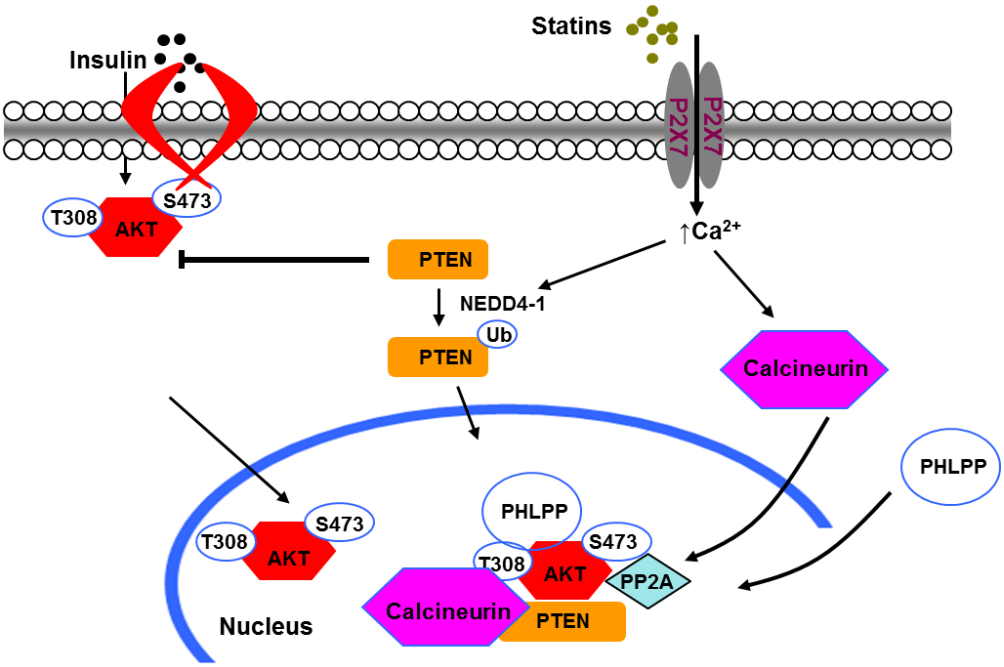

Figure 7: Statins or ATP induce a phosphatase complex that depletes nuclear pAkt in insulin-stimulated cells.

4.2 PAPER II: SILENCING P110B PREVENTS RAPID DEPLETION OF NUCLEAR PAKT

The p110β subunit in the class IA PI3K family may act as an oncogene and is critical for prostate tumor development in PTEN knockout mice (Jia, Liu et al. 2008). We tested the possible involvement of p110β in a recently described rapid depletion of pAkt in the nucleus. Previous work showed that this down-regulation is induced by extracellular ATP or by statins and is mediated by the purinergic receptor P2X7 (Mistafa, Hogberg et al. 2008; Mistafa and Stenius 2009). In paper I, we found that different Akt phosphatases were involved, including FKBP51 scaffolding protein. Here, in paper II the possible role of p110β was studied in this event. We hypothesized that p110β effect on cell growth is due to its importance for pAkt depletion.

We found that p110β is essential for ATP- or statin-induced nuclear pAkt depletion in MEFs and in several cancer cell lines including prostate cancer cells. ATP, statin or the selective P2X7 agonist BzATP also inhibited cell growth, and this inhibition was not seen in p110β knockout cells.

In paper I we demonstrated that depletion of nuclear Akt correlates with enhanced binding between FKBP51, pAkt and PTEN. In paper II, we found that p110β was necessary for statin-induced changes in binding between FKBP51, pAkt and PTEN. In addition, p110β seems to bind to both FKBP51 and Akt. In several cancer cell lines p110β was crucial for nuclear pAkt down regulation.

Finally, our finding in paper II shows that p110β is essential for the ATP- and statin-induced effects. The finding also provides support for a chemopreventive effect of statins mediated by depletion of nuclear pAkt.

4.3 PAPER III: ATORVASTATIN PREVENTS ATP-DRIVEN INVASIVENESS VIA P2X7 AND EHBP1 SIGNALING IN PTEN-EXPRESSING PROSTATE CANCER CELLS

In paper I and II, the depletion of nuclear pAkt and its significance for chemo-preventive effects of statins were studied. Human prostate studies provide possibly the best evidence that supports an anticancer effects of statins (Roy, Kung et al. 2011). In paper III, nuclear pAkt depletion, mediated by statins, was comprehensively studied in prostate cells. We observed that pharmacologically relevant statin concentrations depleted nuclear pAkt in PTEN expressing prostate cancer cells. In line with paper I and II and earlier reports, the effect was rapid and mediated by P2X7 receptor (Mistafa, Hogberg et al. 2008). In paper I, nuclear localization of phosphatases (PTEN and PHLPP) and PCNA were also seen. We conclude that prostate cancer cells responds in the same manner as A549 cells (paper I) to statin-induced nuclear pAkt depletion.

In genetic studies, EHBP1 has been correlated with aggressive prostate cancer (Gudmundsson, Sulem et al. 2008) and its role in rapid insulin-dependent trafficking is also known (Guilherme, Soriano et al. 2004). In this investigation, we found that EHBP1 is crucial for nuclear depletion pAkt. EHBP1 involvement was additionally supported by the fact that enhanced nuclear EHBP1 was seen after statin treatment. As well as EHBP1 and Akt, P2X7 is also shown to influence invasive growth (Jelassi, Chantome et al. 2011). We observed that statin treatment decreased the invasiveness of prostate cancer cells as expected (Brown, Hart et al. 2012). EHBP1, which is already shown to correlated with proteins implicated in invasiveness (Jovic, Naslavsky et al. 2007), affected cellular invasiveness.

The rapidity of EHBP1 and nuclear pAkt depletion tended to exclude a role of mevalonate in statin-inhibited invasiveness in PTEN-expressing DU145 cells, even though a role for mevalonate in statin-inhibited invasiveness in PTEN null PC3 cells has been reported (Brown, Hart et al. 2012). It is important to note that in that study, the statin treatment was as long as 48 h. Anyway, we found that in DU145 cells mevalonate did not block, but increased the invasive effect of statins. EHBP1 siRNA enhanced the invasiveness in both DU124 and PC3, but

the statin effect on invasiveness was EHBP1-dependent only in PTEN expressing DU145, indicating that different signaling pathway are involved in anti-invasive effect of statins in the two cell lines.

The observed difference in cellular responses led us to study the involvement of yet another protein implicated in translocations and invasiveness (Qin, Xie et al. 2009). We found that P-Rex1 siRNA increased the invasiveness in DU145 cells, while it decreased in PC3 cells. This finding was correlated with invasion markers, MMP2 and MMP9. P-Rex1 seems to be involved in similar manner as EHBP1. In addition, co-localization between P-Rex1 and EHBP1 was also seen, and further co-localization was also seen between PTEN and P-Rex1 in DU145. The fact that PC3 cells lack PTEN, the P-Rex1 binding partner, can possibly explain the fact that statin affected invasiveness differently in DU145 and PC3 cells (Fig. 8).

The natural agonist of P2X7, ATP, has been shown to enhance cellular invasiveness and also affect invasion markers (Zhang, Gong et al. 2010). Our findings confirmed this previous report and further we found that statins inhibited ATP-driven invasiveness and that ATP affects co-localization of EHBP1 and P-Rex1 differently from statin. Together these data indicate that statins can modulate P2X7-induced invasiveness by affecting EHBP1 and P-Rex-1.

To further support a role for P2X7 in statin-induced aggressive prostate cancer prevention, genetic analyses were performed. Significant correlation between SNP rs3751143 (located in P2RX7 gene) with prostate cancer was seen (odds ratio = 0.86, P = 0.044). To put it in other words, the analyses clearly showed that the minor allele is significantly associated with less aggressive prostate cancer (Gleason <7 and stage A/B), and the major allele with a more aggressive prostate cancer (Gleason ≥7 and/or stage C/D). To our knowledge, this is the first time that an association between rs3751143 and cancer is reported. This finding has a number of implications. Firstly, it supports the argument that P2X7 enhances growth of tumor cells (Adinolfi, Raffaghello et al. 2012). Secondly, in breast cancer cells P2X7 promotes invasive capacity (Jelassi, Chantome et al.

2011). Finally, it is argued that ATP boosts the invasiveness of prostate cancer cells (Zhang, Gong et al. 2010).

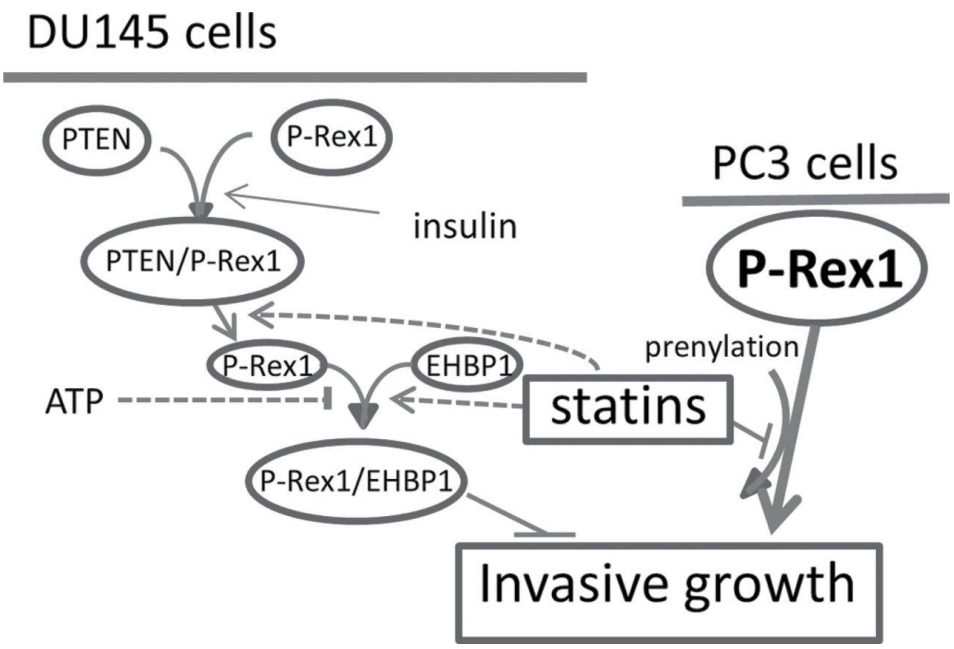

Figure 8: Involvement of different signaling pathways in PC3 and DU145 cells induced by statins via the P2X7 receptor.

4.4 PAPER IV: PHOSPHATASE AND TENSIN HOMOLOG DELETED ON CHROMOSOME 10 (PTEN) AND PH DOMAIN AND LEUCINE-RICH REPEAT PHOSPHATASE (PHLPP) CROSS-TALK IN CANCER CELLS AND IN TRANSFORMING GROWTH FACTOR B-ACTIVATED STEM CELLS.

In Paper I we implicated PTEN, PHLPP1 and 2 and other phosphatases in protein complexes which lead to depletion of nuclear pAkt. Our data suggest that all of these phosphatases are essential for the statin-induced effect on nuclear pAkt. pAkt and its downstream targets were unaffected by statins in PTEN deficient LNCaP cells. However, the statin-induced down-regulation of nuclear Akt was restored when cells were transfected for PTEN.

In paper IV, we studied whether transfection of PTEN in other PTEN deficient cells affects pAkt depletion in a similar manner as in LNCaP cells. However, in these cell lines PTEN transfection did not restore the capacity of statins to deplete nuclear pAkt. Instead, we found that in PC3 cells the PTEN transfection lowered levels of PHLPP2 and PHLPP1. When the cells were transfected for both PTEN and PHLPP2 the level of a third Akt phosphatase, PHLPP1, was reduced. We investigated if this crosstalk phenomenon is applicable in PTEN expressing cells. The crosstalk between PHLPP and PTEN was further studied in PTEN expressing 22RV1 cells and it was found that overexpression or silencing of PHLPP1 and 2 levels affects the levels of PTEN. A similar crosstalk was also observed between PHLPP1 and 2. To evaluate if this crosstalk occurs in non-transformed cells PTEN, PHLPP1 and PHLPP2 were overexpressed in normal prostate RWPE-1 cells, mouse embryonic fibroblast (MEFs) cells and rat liver TRL 1215 cells. No phosphatase crosstalk was observed in any of these cells. In view of this finding we asked if PTEN and PHLPPs regulate each other transcriptionally or/and epigenetically. RT-PCR analyses showed that PTEN overexpression reduced PHLPP mRNA in PC3 cells and PHLPP2 overexpression reduces PTEN mRNA and PHLPP1 in 22RV1 cells. Both PTEN and PHLPP have been shown to be regulated epigenetically by miRs (Yang, Kong et al. 2008; Beezhold, Liu et al. 2011; Liu, Wu et al. 2012). In PTEN-overexpressing PC3 cells, miR-190 was significantly increased, and a similar pattern was seen for miR-214 in PHLPP2-overexpressing 22RV1 cells.

Furthermre, the inhibition of miR-190 and miR240 blocked the phosphatase crosstalk, confirming the involvement of miRs (Fig 9). This data supports the notion that crosstalk-induced repression is due miR changes. The C-terminus of PTEN is essential for its binding capacity (Fan, He et al. 2009) and the phosphatase activity of PTEN (Odriozola, Singh et al. 2007). In our model, overexpression of C-terminus-deleted PTEN (CD-PTEN) did not affect PHLPP level, showing the importance of the C-terminus of PTEN for crosstalk. No transcriptional or epigenetic changes were seen in non-transformed cells after phosphatase overexpression.

In both cancer and embryonic stem cells, miR-214 has been shown to regulate polycomb group (PcG) of proteins (Juan, Kumar et al. 2009). Binding between members in PcG proteins, e.g. Bmi1 to Evi1 repress PTEN (Yoshimi and Kurokawa 2011). The level of PcG proteins are elevated in PTEN-null aggressive prostate cancer. Furthermore, Bmi1 has been shown to inhibit the growth of PTEN-null aggressive prostate cancer (Lukacs, Memarzadeh et al. 2010). Therefore, we tested if PcG proteins were involved in the crosstalk. We found that binding between chromatin and Evi1 was enhanced after PHLPP2 transfection in 22RV1 cells. This is in agreement with the fact that Evi1s binding to PcG proteins has been shown to repress PTEN levels (Yoshimi, Goyama et al. 2011). Increased binding between Evi1 and Bmi1 was also seen in PTEN-overexpressing PC3 cells. There is evidence that activation of PcG proteins suppress genes by DNA methylation. After using a DNA-methylation inhibitor no crosstalk between PHLPP2 and PTEN was observed. This result appears to confirm that there are epigenetic mechanisms involved in the phosphatase crosstalk.

A major characteristic of this crosstalk is that it occurs only in cancer cells. Other have shown that PTEN depletion induces epithelial-mesenchymal transition (EMT) (Leslie, Yang et al. 2007). This brings us to the question of whether there is phosphatase crosstalk in stem cells undergoing EMT? Some key player of the crosstalk e. g. Bmi1 and miR-214 have increased activity in a number of cancer stem cells (Liu, Dontu et al. 2006). Moreover, Bmi1 is associated with the

regulation of both normal and cancer stem cells (Molofsky, Pardal et al. 2003; Park, Qian et al. 2003). It is also known that TGFβ-1 activates EMT (Thiery, Acloque et al. 2009). In WPE prostate stem cells EMT was activated by TGFβ-1. Several EMT characters were seen such as changes in morphology, increasing cell proliferation and invasiveness. Interestingly, when we tested the cells for the crosstalk, we found phosphatase crosstalk in the TGFβ-1-treated WPE stem cells but not in non-treated cells. This observation indicates that activation of phosphatase crosstalk in prostatic stem cells is part of EMT.

The level of PTEN has been shown to affect invasive properties (Shukla, Maclennan et al. 2007). Other factors such as Bmi1, miR-214 and Evi1 are also associated with invasiveness (Penna, Orso et al. 2011). In time-response experiments PTEN overexpression affected MMP9 and MMP2. PTEN overexpression decreased MMP level until 40 hours of transfection, but after 1-3 days post transfection the MMPs and pAkt levels enhanced again. This observation was confirmed, for some specific time points, by invasion assay. Similar observations were seen when the cells were transfected for two phosphatases, while transfection for all three phosphatases decreased the invasiveness even 3 days after the transfection. These data suggest that crosstalk enhances the invasive capacity of PC3 cells.

Previously, in paper I, II and III the purinergic P2X7 receptor has been implicated to act as a key player in pAkt nuclear depletion mediated by phosphatases. To investigate the possible role of purinergic receptors we used HEK293 cells. No crosstalk was seen in the HEK293 cells overexpressing empty vector, or in HEK293 cells heterologously expressing P2X7. However crosstalk occurred in HEK293 cells expressing P2X4. We also noted that, siRNA P2X4 inhibited the crosstalk in both PC3 and TGFβ-1-treated WPE cells. From these findings, we concluded that P2X4 is crucial for the phosphatase crosstalk.

This suggests that if P2X4 is important for the crosstalk and if invasiveness is crosstalk mediated, the role P2X4 in invasiveness is interesting to study. Invasion assay analyses showed that in both TGFβ-1 treated PC3 and WPE cells, P2X4 inhibitor or P2X4 siRNA inhibited the TGFβ-1-induced invasiveness. This

finding was confirmed by Western blotting experiments. Moreover, the involvement of P2X4 was again confirmed when BAPTA-AM, an inhibitor of calcium signaling was used. P2X4 activity is dependent on Ca^{2+} (Glass, Loesch et al. 2002), and using the calcium signaling inhibitor abrogated the crosstalk. We can assume that in TGFβ-1 driven invasiveness of PC3 and WPE stem cells, P2X4 plays a key role.

P2X4 involvement suggests a link to processes such as cell death and repair (Freeman, Bowman et al. 2011), we studied regenerative preneoplastic rat liver lesions. Female Sprague-Dawley rats were treated for 11 weeks with diethylnitrosamine (Silins, Hogberg et al. 2006) which leads to induction of lesions. Involvement of cell damage-repair cycles, stem cell origin (Sell and Leffert 2008) and TGFβ-1 signaling has been shown in such lesions (Takahashi, Shibutani et al. 2008). Interestingly, the levels of the phosphatases were expressed differently between preneoplastic and normal tissue and suggested an involvement of an active crosstalk.

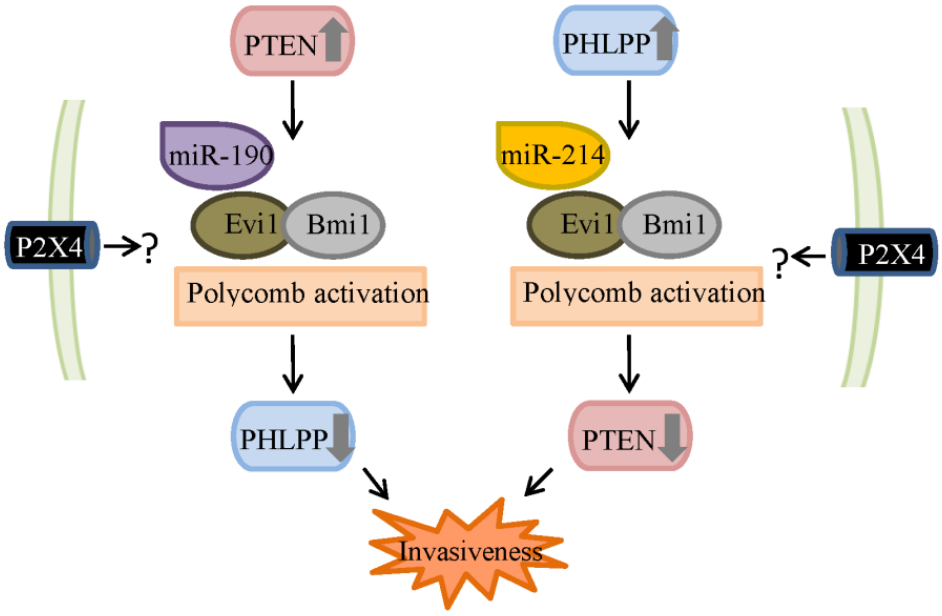

Figure 9: Crosstalk between PTEN and PHLPP in cancer cells or TGFβ-1 activated stem cells, involving miRs, polycomb group of proteins and P2X4.

5 CONCLUSIONS

The PI3K/Akt signaling pathway is frequently up-regulated in human cancers. Akt is an important molecule in cell survival pathways. Akt is able to induce protein synthesis pathways, and is therefore a key protein involved in general tissue growth. Akt is negatively regulated by lipid and protein phosphatases. Previously, our group has demonstrated rapid nuclear pAkt depletion, mediated by anti-cancer drugs and by cholesterol lowering statins (Roudier, Mistafa et al. 2006).

The experiments presented in this thesis were aimed to map out the mechanism behind rapid nuclear pAkt depletion. In general, our results show that cholesterol-lowering drugs, statins, or extracellular ATP, induced a complex and coordinated response in insulin-stimulated A549 cells leading to depletion of nuclear pAkt. It involved protein/lipid phosphatases PTEN, PHLPP1 and -2, PP2A and calcineurin. Our results also indicate that this effect was mediated through P2X7 receptor.

The described complex leading to pAkt depletion was further investigated and a possible role for p110β was elucidated. We conclude that p110β is essential for nuclear pAkt depletion in MEFs and in several cancer cell lines, including prostate cancer cells.

The mechanism behind the pAkt depletion has been a central focus of this thesis. We concluded that both EHBP1 and P2X7 are linked to aggressive prostate cancer and that rapid nuclear pAkt depletion may affect invasiveness. Another interesting observation is that pharmacological concentrations of statins decrease nuclear pAkt in non-transformed prostatic cells, suggesting that an anticancer effect of statins might be mediated by the Akt pathway.

Yet another interesting aspect of this thesis is the delineation of a crosstalk between PHLPPs and PTEN. This crosstalk was seen in cancer cells and TGFβ-1 activated prostate stem cells, and had an impact on cellular invasiveness. The

P2X4 receptor was shown to be mediator of the crosstalk and epigenetic and transcriptional factors were implicated.

Finally, the comprehensive downstream consequences of this crosstalk remain to be explained. This crosstalk phenomenon seems to reflect a very complex regulatory circuitry, effects which might explain why cancer therapeutic efforts so often fail. Our findings, in this thesis, may provide data that, taken together, gives a first picture of a novel feedback system that may act to protect cancer cells against therapeutics.

6 SIGNIFICANCE

Understanding cellular pathways is a key step for the development of better chemoprevention and therapies to treat cancer and tumor cells. As mentioned, the PI3K/Akt signaling pathway is up-regulated in a variety of human cancers. We hope that this study will increase the understanding of how Akt signaling is regulated. Our results may lead to broader knowledge about cancer development but also about treatment and prevention.

We think that we have been able to elucidate some complex cellular events of importance for cancer progression. We hope that its potential importance, its complexity and its interaction with signaling pathways previously associated with other biological processes than cancer, should be an inspiration for other researchers.

Compounds with cancer preventive properties are strongly desired. Through the experiments performed in studying Akt pathway, we have also explained, although not comprehensively, a possible chemo-preventive effects of statins in prostate cancer cells. A profound understanding of the basic mechanisms behind different anticancer drugs would help to reduce complications such as chemo-resistance and may also generate ideas about drug interactions that can improve therapy.

7 FUTURE PERSPECTIVES

The data presented in this thesis clearly highlights the involvement of the Akt pathway and its regulation for multiple cellular endpoints such as invasion and proliferation, events which are of high importance for cancer progression. Remarkably, the precise mechanisms and sources of invasion/proliferation signals are poorly understood. The role of Akt and its phosphatases is an interesting approach for improving our understanding. However, in this thesis, we did not provide a full characterized mechanism, and more mechanistic studies are needed. For example, Akt may interact with other signaling pathways e.g. MAPK or Wnt signaling which was not studied here. We speculate that a broader view may be beneficial since Akt signaling seems to be safeguarded in neoplastic cells by back-up loops that possibly can be targeted in successful therapy of tumors.

We have been able to show that statins deplete nuclear pAkt though P2X7 receptor. It is shown that P2X7 and P2X4 interact with each other in their natural environment and our findings show that even 0.1mM ATP affected our model system. Earlier papers suggest that P2X4 is involved in those findings. Integration between statins with P2X4 and P2X7 can be an interesting approach to study in more detail.

We have also reported differences between PC3 and DU145 cells in response to statins, but the mechanism behind this difference is unclear. In data not included in this thesis, crosstalk between PTEN and P-Rex1 has been seen. More detail studies of this interrelationship and their correlation to P2X7, statins, extracellular ATP and EHBP1 may identify biomarkers reflecting prostatic preneoplastic lesions that has the properties to develop into aggressive cancer.

One of the weaknesses of the studies presented in this thesis is the shortage of *in vivo* experiments. Several aspects need at this stage to be examined *in vivo*. Genetically engineered mice injected subcutaneously with prostate cancer cells might be an experimental design for the study of the role of P2X7 – Akt in prostate cancer development.

Some of the studied proteins in this thesis are associated with prostate cancer through SNP studies, but some remain to be analyzed. SNPs on P-Rex1 and P2X4 may provide more complete evidence of a role of a putative P2X4-P2X7-EHBP1-Akt-P-Rex1 in prostate cancer. As we have shown, this pathway is affected by statins. SNP studies on P-Rex1 and P2X4 may further support the relevance of the indicated chemo-preventive effect of statins.

8 ABSTRAKT PÅ SVENSKS

Cancer är en ledande dödsorsak världen över. PI3K/Akt-signalvägen är uppreglerad i cancer Och Akt (kallad även för PKB) är en viktig signal-molekyl som verkar för cellöverlevnad. Aktivt Akt eller fosforylerat Akt (pAkt) inducerar proteinsyntes och är ett nyckelprotein när det gäller vävnadstillväxt. Flera fosfataser (både lipid- eller protein-fosfataser) har visats kunna hämma Akt-signalvägen. Nukleär Akt är nödvändig för dess aktivitet och funktion.

Tidigare hade man visat att de kolesterolsänkande och anti-carcinogena läkemedelen statiner snabbt tömmer pAkt från kärnan. Vi har fokuserat på mekanismen bakom den snabba pAkttömningen i kärnan. I den första studien kom vi fram till att i insulin-behandlade A549-celler inducerar statiner eller extracellulär ATP bildning av ett proteinkomplex som sannolikt orsakar nukleär tömning utav pAkt. Flera protein/lipid- fosfataser var involverad såsom PTEN, PHLPP1 och 2, PP2A och calcineurin. Den purinerga receptorn P2X7 identifierades som en mediator av denna effekt.

I den andra studien studerades den snabba nukleära pAkt tömning vidare. En PI3K-subenhet, p110β, och dess möjliga roll undersöktes. Denna subenhet har varit associerad med aggressiv prostatacancer, och våra resultat på musembryofibroblaster och på cancerceller visade att p110β är nödvändig för nukleär pAkt-tömning.

EHBP1 och P-Rex1 har involverats i proteintransport, rekrytering av proteiner till kärnmembranen. De är dessutom associerade med aggressiv och invasiv prostatacancer. I den tredje studien, fann vi att P2X7 är korrelerad med aggressiv prostatacancer och att den P2X7-beroende pAkt-kärn-tömningen i sin tur är beroende av . Även P-Rex1 spelade roll. Vidare fann vi att farmakologiskt relevanta koncentrationer av statiner minskade pAkt i kärnan på normala prostataceller. Detta tyder på att statins anticancereffekt kan bero på en hämning av Akt-signalvägen.

I den fjärde studien karaktäriserade vi en ”crosstalk” mellan Akt-reglerande PHLPP och PTEN. Denna ”crosstalk” gör attDessa båda fosfataser balanserar varandras uttryck i cancerceller och TGFβ-1-aktiverade prostata-stamceller. Detta hade betydelse för cellinvasivitet. P2X4-receptornidentifierades som kritisk för att ”crosstalken” mellan Akt-fosfataserna utvecklades. Förutom P2X4, är både genetiska (transkription) och epi-genetiska faktorer involverade.

Sammanfattningsvis, dessa studier visar en ny mekanism som leder till nukleär pAkt-tömning. Vi har även visat att P2X7-EHBP1-Akt kan vara involverad i prostatacancer-utveckling och att Akt-hämningen kan påverka cancercellernas kapacitet att bli invasiva. En ”crosstalk” mellan flera Akt-hämmande enzymer kan försvåra pAkt-tömning och bidra till cancercellernas kapacitet till invasivväxt.

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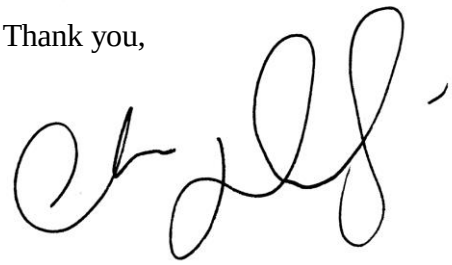

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